Art. XII.—*Researches into the Serological Diagnosis of Contagious Pleuro-Pneumonia of Cattle.*

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(Communicated by Professor H. A. Woodruff.)

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Introduction.

Contagious pleuro-pneumonia was introduced into Australia by the importation into Victoria of an infected cow from Great Britain in 1858. Soon after its introduction into Australia the disease spread with alarming rapidity, and in a very short time it had become disseminated through every State in the Commonwealth.[1]

At the present time the disease exists in each of the Australian States, with the single exception of Tasmania, which, owing to its geographical situation, has been able, by the adoption of rigid quarantine restrictions, to prevent introduction from the mainland.

In Victoria an active policy has been adopted by the State Veterinary Department, in dealing with outbreaks; the measures adopted being strict quarantine of infected and incontact herds, the slaughter of all visibly infected animals, isolation of all doubtful cases, and prophylactic immunisation by inoculation of virus subcutaneously in the tail of all contacts.

That these measures have proved inadequate to free the State of pleuro-pneumonia, and keep it free from the disease is evidenced by the occasional outbreaks which occur from time to time in various portions of the State. On the other hand, their application has been attended with a considerable amount of success, and any relaxation of them is immediately followed by an extension of the disease throughout the State.

As an instance: During the late war, owing to the absence on active service of the majority of the members of the State

1. W. T. Kendall (1913), "Notes on the Early History of the Veterinary Profession in Victoria." Report of the 14th meeting of the Australasian Association for the Advancement of Science, p. 704.

Veterinary service, it became necessary for the Department of Agriculture to modify and relax the usual method of dealing with outbreaks of contagious pleuro-pneumonia. It is a significant fact, that during that war period, the disease became more prevalent throughout Victoria than it had been for many years.

As far as Victoria is concerned, the difficulties of control and eradication are enormously increased by the border traffic in animals from neighbouring States, and the impossibility of recognising, by clinical examination alone, the presence of carriers of latent infection.

The records obtained over a number of years in Victoria show the large percentage of outbreaks of the disease, which owe their origin to the unsuspected introduction of a carrier of the infection into a healthy herd. The diagnosis and destruction of these so-called "recovered animals," carriers of a potential infection, which readily becomes actual when they come in contact with susceptible animals, is therefore a matter of prime importance in successfully eradicating the disease from any particular State.

In certain European countries, notably Great Britain, and in the U.S.A., the disease has been eradicated by adopting the procedure of wholesale slaughter of all the animals concerned in each and every outbreak. This procedure, whilst economically justifiable in those countries with our present knowledge of the disease, is at the same time unscientific and costly, since it necessitates the destruction of a very large number of healthy cattle in order to attain its object.

The difficulties of adopting such a course of stamping out the disease in Australia at the present day are obvious, owing to the large area over which the disease has spread, the scattered nature of the outbreaks, the number of cattle, the slaughter of which would be involved in such stamping out process, the enormous cost of compensating the owners of the slaughtered animals, and also owing to the existence of the disease on stations situated in the more remote parts of Australia, where it would be impossible to muster all the cattle on the particular property at any one time.

The variable incubation period, which may be anything from 10 to 30 days, and the fact that an animal affected with the disease, but not showing recognisable clinical signs of such infection, may be an actual infective agent, renders the task of localising outbreaks exceedingly difficult. The role played in the dissemination of the disease by so-called "recovered animals,"

adds a further complication to the effective eradication of the disease from any particular State. These "recovered animals" are capable of retaining the infective agent in a latent form for at least several months, and the unsuspected introduction of such animals into healthy herds often provides the starting points of fresh outbreaks.

Thus, apart from animals in which the disease can be recognised by the clinical symptoms alone, the disease is mainly propagated by "carriers" (so called "recovered" animals), or by affected animals in which the usual clinical signs of the disease are not apparent. The importance of eliminating such animals as early as possible from herds in which the disease has appeared, and the difficulty of attaining the desired object when one has recourse only to the usual clinical methods of diagnosis, made it essential that some more searching and reliable diagnostic method should be elaborated, which could be applied to such cases, in order to detect them. It was mainly with the object of attempting to arrive at such a diagnostic method that this present research work was undertaken.

I desire to express my grateful appreciation and thanks to Dr. S. S. Cameron, Director of Agriculture, Victoria, and to Mr. W. A. N. Robertson, B.V.Sc., Chief Veterinary Officer, Department of Agriculture, Victoria, for the opportunity and assistance they have granted me to pursue the study of contagious pleuro-pneumonia uninterruptedly for close upon twelve months.

To the staff of the Live Stock Division, Department of Agriculture, Victoria, I am indebted for the collection of blood samples from infected and non-infected animals, and for post-mortem records of the animals supplying the various sera tested.

To Professor H. A. Woodruff, Director of the Veterinary Research Institute, Melbourne University, I desire to express my grateful appreciation and thanks, and acknowledge my indebtedness for the assistance rendered to me by himself and the staff of the Research Institute, during the course of this research work. The work has been carried out in the laboratories of the Veterinary Research Institute, and the materials of the laboratories, and the assistance of the staff have been placed freely at my disposal throughout.

To Dr. L. B. Bull, Deputy Director, South Australian Government Laboratory of Pathology and Bacteriology, I am indebted for much kind advice and criticism of the earlier work on Agglutination and Complement Fixation.

I am also deeply grateful to the trustees of the Walter and Eliza Hall Research Fund, for the appointment as "Walter and Eliza Hall Research Fellow in Veterinary Science," and for providing from their fund the moneys required to cover the expenses incidental to this research work.

Historical Résumé.

The earliest conception of the etiology of pleuro-pneumonia was that cold was the primary cause operating in the production of the disease.

In 1852 Willems (20)[2] as a result of a number of observations and experiments, demonstrated that the disease was contagious, and that healthy animals had to come into close contact with diseased animals before the disease would spread from one to the other. He further demonstrated that, in order that the disease could spread, it was necessary for contact to be between living animals. The disease was not spread when healthy animals were exposed to contact with the carcase of an animal which had died from contagious pleuro-pneumonia. His experiments with pleuritic serosity are of special interest, as they mark the commencement of a system of immunisation against the disease, which system, with but very slight modifications, is extensively practised at the present day. Willems found that if a small quantity of pleuritic serosity, taken from an animal affected with pleuro-pneumonia, was injected subcutaneously behind the shoulder of a healthy animal, there followed, after an incubation period varying from 8 to 15 days, a firm swelling, later becoming fluctuating, hot and painful, which then rapidly increased in size, and gave rise to an invading oedema of the whole of the connective tissue in the region of the inoculation. This swelling contained a large quantity of clear amber-coloured serosity, a small quantity of which injected into another healthy bovine animal behind the shoulder gave rise to a similar swelling at and around the site of inoculation. Death of the experimental animals almost invariably followed these experimental inoculations behind the shoulder, but when inoculations with the same materials were made subcutaneously in the tail, a few centimetres from the tip, there followed a mild reaction, with some swelling of the tail, which usually subsided in the course of 15 to 21 days, but which occasionally terminated in a more or less extensive

2. Reference is made by numbers to "Literature Cited," pp. 63-65.

necrosis of a portion of the tail. These benign reactions, he announced, were productive of a marked degree of immunity against natural infection with the disease At no time, as a result of these inoculations, either behind the shoulder, or in the tail, was he able to demonstrate that they gave rise to a definite pleuro-pneumonia of the lungs, similar to that seen in animals naturally infected. He noticed, however, that the swelling produced as a result of the subcutaneous injection of lymph behind the shoulder had many features histologically comparable with the condition set up in the lungs by natural infection.

In 1883 Pasteur (16) carried out some experiments with the so-called "lymph" (pulmonary and pleuritic exudate), and he declared that the lymph contained pure virus, which could not be cultivated on ordinary media.

"Sussdorf (1879), Bruylants and Verriest (1880), Putz (1881), Himmelstoss (1884), Lustig (1885), Poels and Nolen (1886), isolated and described various microbes without establishing their specificity." (12)[3]

In 1895 Arloing (1) isolated from cases of pleuro-pneumonia a short non-motile bacillus, which he named the pneumo-bacillus-liquefaciens-bovis, and which he claimed was the causal organism of pleuro-pneumonia. Pure cultures of this organism, according to Arloing, when introduced intra-pulmonarily into healthy animals, were capable of producing the characteristic lung changes seen in naturally infected cases of pleuro-pneumonia, while subcutaneous injection of pure culture into susceptible animals produced immunity. Arloing's experiments are open to a considerable amount of criticism, owing to the technique he employed, and although his experiments were repeated by others, his announced results could not be confirmed.

In 1898 Nocard and Roux (13), by means of an ingenious experiment, succeeded in artificially cultivating the virus of pleuro-pneumonia in bouillon contained in collodion sacs, which were inoculated with a trace of pulmonary serosity from an animal affected with pleuro-pneumonia, and were then embedded in the peritoneal cavities of rabbits. After 15 to 20 days the bouillon, which at the commencement of the experiment was perfectly limpid, showed a faint opalescence, and thereafter the rabbits became emaciated. The contents of control sacs similarly treated, but not inoculated with serosity remained perfectly clear and

3. P. 449.

sterile, and the rabbits remained healthy. There were confined in the sacs containing the opalescent medium no bacteria capable of cultivation on the ordinary laboratory media. Under microscopic examination, with a high magnification (1500-2000 diameters), and with an abundant illumination, there was observed in the opalescent culture medium a number of very small and refringent points, so small that their individual structure could not be accurately determined.

Shortly after the discovery of the causal organism of contagious bovine pleuro-pneumonia by Nocard and Roux, the disease engaged the attention of a number of scientists in various parts of the world. In 1900 Dujardin-Beaumetz (6) published his thesis on the isolation and cultivation of the organism discovered by Nocard and Roux, which he showed could be grown *in vitro* in a special medium, consisting of Martin's peptone bouillon, with the addition of serum. The organism was capable of traversing Berkfeld and Chamberland F. filter candles provided the virus or culture was suitably diluted prior to filtration. On the contrary, the Chamberlain B. filter candles formed an impassable barrier to the organism, irrespective of the dilution employed. He also described the characters of the culture in special broth, and on solid media, and was able to provoke in cattle, by subcutaneous inoculation of pure culture in the trunk, a typical oedematous engorgement corresponding in all its appearances to that following the inoculation of lung virus into similar animals in similar situations.

In 1906 Dujardin-Beaumetz (7) succeeded in producing, with pure cultures of the organism in broth plus sheep's serum, and in broth plus horse serum, similar lesions in sheep and goats to those observed following the inoculation of culture in broth plus ox serum into cattle.

In 1910, Borrel, Dujardin-Beaumetz, Jeantet, and Jouan (4) described in detail the morphology of the organisms they had isolated from pure cultures. They remarked upon the polymorphism of the organisms in the preparations examined and described filamentous forms, chains, granules, round forms, ovoid forms, and pseudo-vibrion filaments. Asteroid forms in particular were described, and they suggested the name "Asterococcus mycoides" for the organism.

Bordet, in 1910 (2), also published the result of his observations concerning the morphology of the organism of contagious pleuro-pneumonia. He described granules and filaments re-

sembling spirilla and spirochetes, which originated from single granules.

Following closely on the work of Borrel, Dujardin-Beaumetz, Jeantet and Jouan, and on that of Bordet, Martinovski (8), in 1911, published an account of his observations of the organisms in cultures and in sections of diseased tissue. He also remarked upon the polymorphism of the organism, and after having described various forms, some of them similar to some of those observed by Borrel, he concludes that "the study of the pleuro-pneumonia microbe in the tissues of infected animals, and in the cultures, allows us to classify the microbe of Nocard and Roux in the group of cocco-bacilli, so that the name "Coccobacillus mycoides peri-pneumonic" would suit it well, as it indicates all the properties of this singular organism."[4]

K. F. Meyer (1909) (9) described in detail the pathological changes which occur in the lungs and other tissues in naturally infected cases of contagious pleuro-pneumonia, and also the changes which occur in tissues following a subcutaneous injection of virus in the dewlap or in the tail. He states:—"It is interesting to demonstrate that whichever tissue may have been the seat of injection of pleuro-pneumonia virus, it always shows exactly the same changes as have been described as being found in the interstices of the lungs under natural infection."[5]

It is of passing interest to note that Meyer, in referring to the complications such as necrosis of the tail, peritonitis, etc., which sometimes follow the inoculation of virus, remarks:—"It has been stated that contamination of the vaccine is the cause of complications. I quite disagree with such a notion, for working with absolutely pure cultures, and taking all aseptic precautions, we yet cannot avoid having losses. Individual disposition, or weakening of the constitution form the cause of the misfortune."[6]

Boynton (1912) (5) has shown, as a result of a close study of the muscular changes brought about by intermuscular injection of infected material into otherwise healthy bovines, that the lesions occurring in the muscles following such injections are histologically comparable with the lesions occurring in the lungs in cases of the disease naturally acquired.

4. P. 917.
5. P. 153.
6. P. 153.

It was to be expected that, following on the isolation and successful cultivation of the causal organism of contagious pleuro-pneumonia, attempts would be made to apply the usual serological reactions, such as agglutination, precipitation, and complement fixation, for the diagnosis of the disease in the living animal.

Dujardin-Beaumetz (1900) (6), after experiments with culture and serum from immunised bovines, announced that the "serum of hyper-vaccinated animals is not bactericidal, and agglutination is not able to be of any use in the diagnosis of pleuro-pneumonia." However, in 1906 (7) he found that massive intravenous injections of pure culture in Martin's broth plus horse serum into horses gave rise to agglutinins in the horse serum, which could be demonstrated in dilutions up to 1-50. Further he demonstrated that this same serum would give a precipitin reaction when combined with the serum of experimentally inoculated bovines, and he suggested that, although as a serodiagnostic method it was delicate in performance, it might be of some use in order to confirm clinical diagnosis in certain chronic cases of the disease.

Schochowsky in 1912 (18) published the results of his work on complement fixation in relation to pleuro-pneumonia. He concluded that it was impossible to obtain a reliable complement fixation reaction, and that complement fixation had no value as a serodiagnostic method for contagious pleuro-pneumonia. This view was also expressed, though not so definitely, by Poppe (1913) (17), who obtained unsatisfactory and contradictory results with his work on complement fixation. He found, however, that a precipitation test (Fornet's ring reaction), gave fairly reliable results, but that sera had to be specially selected to act as precipitinogen. He concluded that the test could be used with advantage for the diagnosis of doubtful cases where the clinical evidence was insufficient.

K. F. Meyer (1914) (10), in a review of the filterable viruses in general, refers briefly to the more important work which has been done in recent years on contagious pleuro-pneumonia. He points out that "the study of the filterable viruses is attended with considerable technical difficulties, and the experimental results obtained depend largely on the ingenuity of the experimenter."[7] Referring to the complement fixation test in pleuro-pneumonia, he states:—"In some unpublished experiments the author found the complement fixation tests very unreliable for

7. P. 267.

the demonstration of an existing immunity in contagious pleuropneumonia of cattle. Only in one naturally infected animal could immune bodies be detected."[8]

Panisset (1914) (15) has also published a summary of our present knowledge of the filterable viruses in general, and in doing so, has covered much of the ground covered by Meyer in his review. It is interesting to note that Panisset in his summary states:—" L'immunisation contre les virus filtrants ne semble pas proceder des memes principes que l'immunisation contre les bacteries. Les phenomenes humoraux qui accompagnent l'etat d'immunite sont peu marques (agglutination, precipitation) ou manquent complement, on ne les observe bien que dans la peripneumonie qui a beaucoup d'egards est une maladie bien differenciee. Cependant la fixation des sensibilisatrices du serum est assez souvent observee."[9]

Cultures.

The foregoing chapter is a short resume of the literature at my disposal when this research work was commenced. Although it seemed probable that any attempt to apply the agglutination or complement fixation tests for the diagnosis of contagious pleuropneumonia would not be successful, it was decided to proceed with the research work, more especially because no such work had been previously attempted in Australia. Accordingly, on 4/10/19, a commencement was made by obtaining virulent lung serosity from a naturally infected cow which was killed at the City Abattoirs, Melbourne. A small quantity of this lung serosity was collected under aseptic conditions in sterile Pasteur pipettes, and inoculated direct into ten tubes of Martin's peptone bouillon plus 7.5 per cent. normal ox serum, and placed in the incubator at 37°C. A further quantity of the lung serosity from the same animal was collected in a sterile bottle, and brought to the laboratory. This serosity was used later for an experiment to demonstrate the filter-passing properties of the organism of contagious pleuropneumonia.

Of the ten tubes inoculated direct with virulent lung serosity on 4/10/19, four showed obvious contamination after 36 hours' incubation, and were discarded. Of the six remaining tubes one proved sterile, the other five showed in from 4 to 6 days a faint

8. P. 284.
9. P. 312.

opalescence in the culture medium. This opalescence was extremely slight, and could be best recognised by holding the tubes in such a manner that varying degrees of light fell upon them, at the same time comparing each tube with one or more control tubes of the same medium, which had been incubated, but which had not been inoculated. In order to recognise the presence of this slightly opalescent culture in Martin's broth serum medium it is absolutely essential that each lot of tubes inoculated and incubated should be efficiently controlled by incubating tubes of the same broth serum medium, which have not been inoculated, and comparing them from time to time.

Sub-cultures made into Martin's broth plus ox serum, and incubated at 37°C. showed in 3 to 4 days the same opalescent appearance noted in the primary cultures. This opalescence could still be obtained after several generations of subcultures.

Examination of stained films, made with this opalescent broth, under the microscope with a magnification of 1000 diameters, failed to reveal any recognisable micro-organisms, the material in the film staining as a homogenous mass.

In order to make sure that the opalescence in the broth was due to the growth of micro-organisms, sub-cultures from the broth tubes were made on to Martin's-broth-agar with sterile ox serum added after it had been sloped. On this solid medium very fine colonies developed in four days, at first only recognisable by means of a lens, but by the eighth day they assumed the size of a pin's point. More or less colourless at first, and appearing like drops of dew on the surface of the medium, they later on became slightly opaque. These colonies appeared on the surface of the agar, but were firmly embedded into it, and were dislodged with difficulty. In most cases they had to be dug out of the agar, so firmly were they attached. Several colonies were removed, and stained for microscopic examination *en bloc*. They stained readily with the basic stains, but were decolourised by Gram's method. Although the shape of the colony could be distinguished under the microscope, individual organisms could not be seen with a magnification of 1000 diameters.

Sub-cultures from the Martin's broth tubes were made into Martin's broth tubes containing 1 per cent. of various sugars —saccharose, glucose, maltose, lactose, and the alcohol derivatives—mannite and dulcite. In the mannite and dulcite tubes no growth took place. This experiment was subsequently repeated with other cultures of the organism inoculated into mannite broth

with a similar result. In glucose Martin's broth there is a very definite acid reaction developed. The acid reaction is apparent after the third day of incubation, and increases towards the eighth day, when it apparently attains its maximum, though it is apparent throughout the life of the culture. No gas is developed by the organism in this medium. In maltose Martin's broth there is also a very definite acid reaction produced by the organism, but no gas is developed. In lactose Martin's broth, and in saccharose Martin's broth, there is growth of the organism, but no acid or gas is developed. Tubes of each medium not inoculated, were incubated as controls, and showed an unaltered appearance on comparison with tubes of similar media, uninoculated, and not incubated. The reaction of the organism on cultivation in media containing various sugars can be summarised as follows:—

	Saccharose.	Glucose.	Maltose.	Lactose.	Mannite.	Dulcite.
Acid	- — -	++	- +	- — -	No growth	- No growth
Gas	- — -	—	- —	- — -		

++ Strongly acid.

In order to obtain cultures it is an absolute rule that the culture medium must contain serum. In ordinary broth the organism will not develop. On ordinary nutrient agar the organism will not develop. No growth takes place on gelatine. For all growths obtained, Martin's peptone bouillon has been used as a basis for the medium, and various sera have been tried in various proportions. Growths have been obtained in Martin's broth, to which either ox serum, horse serum, or rabbit's serum has been added. No growths have been obtained in Martin's broth with guinea-pig's serum added, although several such tubes have been inoculated. Growths have been obtained in Martin's broth medium, in which the added ox serum did not exceed 3 per cent. It was found that about the most satisfactory serum content for the culture medium was 7.5 per cent., although very satisfactory growths could be obtained with slightly greater or lesser amounts of serum in the medium.

Filtration Experiments.

Experiments were made to test the filter passing properties of the organism of contagious pleuro-pneumonia. Virus obtained from active lesions in the lungs of cattle, affected with contagious pleuro-pneumonia naturally acquired, which were slaughtered at the City and other Metropolitan Abattoirs, was mixed in varying

degrees of dilution with Martin's peptone bouillon (previously filtered without the addition of serum). The broth and virus were thoroughly mixed, and the mixture was then passed through a Chamberland F. filter at a pressure of 600 mm. of mercury. After all the broth-virus mixture had passed through the filter, the requisite quantity of normal unheated ox serum was filtered through the same filter into the broth-virus filtrate. The resulting filtrate was then placed in the incubator at 37°C.

The whole operation of filtration was usually accomplished in under 1½ hours, but the time depended, of course, on the quantity of material to be filtered. Usually not more than 250 c.c. of broth-virus mixture, followed by the requisite amount of serum was passed through the one filter at the one period of filtration.

A certain amount of difficulty was experienced in demonstrating the filtrability of the micro-organism, and a number of experiments had to be made before it was accomplished. Previous workers [10] have established the fact that, unless properly diluted in a definite proportion (not exceeding 2 per cent.), in a medium without the addition of serum previous to filtration, the virus will not pass through the filter, and a sterile filtrate will result. Although this percentage was not exceeded in any of my experiments (excepting for a special purpose in one series of experiments), a sterile filtrate often resulted, and it would appear that the passage of this organism through Chamberland F. filter candles, is not as easily accomplished as the statements of previous workers, would imply. It is to be regretted that, owing to the war, Berkfeld filters could not be obtained in Melbourne for this experimental work, because the passage of the organism through Berkfeld filters is apparently not attended with the same difficulties as the passage through the finer-grained filters such as the Chamberland F.

In order to prove that absence of growth in the filtrate was not due to changes in the medium brought about by the filtration, the filtrate of one experiment, which had shown no growth on incubation was distributed into four sterile flasks, two of which were then inoculated from a sub-culture of the organism of pleuro-pneumonia, the remaining two flasks being kept as controls

In the two flasks inoculated with the sub-culture, the characteristic opalescent growth was obtained after four days' incubation at 37°C., the two control flasks remained sterile.

10. Nocard, Roux, and Dujardin-Beaumetz.

In order to prove that a culture obtained by primary inoculation of virulent serosity into Martin's broth plus ox serum, was filtrable, the following experiment amongst others was undertaken:—

Two c.c. of a third sub-culture, from a culture which had been obtained by primary inoculation of virulent serosity into tubes of Martin's broth, plus ox serum, at the time that the post-mortem examination was made, was diluted with 200 c.c.s., of Martin's broth, *without serum*, and filtered through a Chamberland F. filter. Fifteen c.c.s. of normal ox serum were then filtered through the same filter into the same filtrate, and the final resulting filtrate was well mixed and distributed into sterile test tubes, and incubated at 37°C. Growth was apparent in 21 days. This experiment—or another similar to it, differing only in the quantities of broth, culture and serum employed (the ratio of culture to broth in each instance never exceeding 1 to 100)—was repeated later with a similar result, excepting that in the second instance growth was not apparent until after 25 days' incubation at 37°C. These experiments prove that the organism obtained in primary cultures by tube inoculations made at the post-mortem is capable of passing through a Chamberland F. filter when the culture is properly diluted with Martin's broth prior to filtration. These experiments have also demonstrated another important fact, namely, that while growth may be apparent in from 4 to 6 days following a primary inoculation of virulent serosity into Martin's broth plus ox serum at a post-mortem, growth is not apparent until from 21 to 25 days after a filtration experiment through Chamberland F. filter candles. The resulting growth in each instance, however, appears to have identical characters.

If the required quantity of serum is added to Martin's broth before filtration of the virulent material, the organisms will not pass through the filters. The Martin's broth used for diluting the virus or culture prior to filtration should first be passed through a Chamberland F. filter in order to facilitate the next filtration when the virus of culture is added. It has been demonstrated by my experiments, that a dilution of $1\frac{1}{2}$ c.c.s. of culture in 100 c.c.s. of previously filtered Martin's broth without serum allowed the organism to pass through the filter, but in another experiment, where 4 c.c.s. of the same culture was diluted with 100 c.c.s., from the same bulk sample of Martin's broth, the resulting filtrate was sterile.

Summary.

The results of the experimental work carried out in order to obtain pure cultures of the organism for use in subsequent work can be summarised as follows:—

(1) Martin's broth (reaction + 10, Eyre's scale), with the addition of 7.5 per cent. of normal ox serum, is the best medium to employ in order to obtain primary cultures of the organism of contagious pleuro-pneumonia. Growth takes place under aerobic conditions of cultivation. The optimum incubation temperature is 37°C.

(2) Good growths can be obtained on subculture into Martin's broth, with the addition of either 7.5 per cent. normal ox serum, or normal horse serum, or normal rabbit serum. The most copious growth on subculture is obtained in Martin's broth plus horse serum, while Martin's broth plus rabbit's serum gives a more copious growth than Martin's broth plus ox serum.

(3) Following primary inoculation of Martin's broth plus ox serum media with virulent serosity, cultures can be obtained after 3 to 4 days' incubation at 37°C.; whereas the same sample of virulent serosity diluted and filtered through a Chamberland F. filter does not give a recognisable growth until approximately 21 days of incubation at 37°C. have elapsed.

(4) The organisms in cultures obtained from primary inoculation of virulent serosity into Martin's broth plus ox serum are capable of filtration through a Chamberland F. filter candle, provided that the culture is first diluted with Martin's broth (without serum) in the proper quantity, i.e., preferably about 1 per cent., but never exceeding 2 per cent. The growth obtained is only recognisable after approximately 21 days following the filtration and incubation of the filtrate at 37°C.

(5) The presence of a growth of the organism in Martin's broth serum media is apparent by the slight opalescence produced in the media after inoculation at 37°C. In order to recognise this opalescence it is imperative that uninoculated tubes of the same broth be incubated along with the inoculated ones. That this opalescence is produced by the growth of a micro-organism can be established by subcultures into other Martin's broth tubes and on to Martin's broth agar, and by the fact that in glucose and maltose Martin's broth media, with the appearance of the opalescence, the reaction of the media is rendered distinctly acid, whereas no change is apparent in Martin's broth, containing sac-

charose or lactose. Animal inoculation ultimately establishes the character of the organisms growing in the media.

Serological Tests.

In order to obtain a supply of cattle serum for subsequent tests, blood was obtained in sterile bottles from naturally infected animals which were slaughtered at the City and various Metropolitan Abattoirs. In each case the carcase of the animal supplying the blood sample was submitted to a post-mortem examination, and lesions of contagious pleuro-pneumonia were demonstrated in the lungs before the blood sample was labelled "positive." Each sample was given a number, and a record kept of the source of each sample.

In addition to these samples, which were mainly collected by myself, I am indebted to the officers of the Stock Diseases Branch of the Victorian Department of Agriculture for several blood samples taken from animals which were killed in the field. No sample was labelled "positive" unless at the time the sample was taken the animal was submitted to a post-mortem examination, and found to be affected with recognisable lesions of pleuro-pneumonia. In this manner it has been possible to obtain a large and representative collection of positive blood samples from several individual outbreaks of pleuro-pneumonia in Victoria.

Blood samples were also taken from cattle whose previous history could be definitely determined, and who were known not to be affected with pleuro-pneumonia, or to have been in contact with affected animals at any time prior to the taking of the blood sample. In addition to these "positive" and "negative" blood samples, blood samples were taken from a few animals which had reacted to an inoculation in the tail of virulent serosity taken from the lungs of an infected animal. These inoculated animals were found on slaughter and post-mortem examination not to be affected with any visible lesions of pleuro-pneumonia in the lungs. Samples of blood were taken from time to time from animals which were being kept and used at the Veterinary Research Institute as experimental animals for pleuro-pneumonia and other cattle diseases. In all cases the blood was taken from the jugular vein, and collected into sterile bottles. The blood was allowed to clot, and the serum to separate off from the clot. The serum was then decanted into another sterile bottle, and diluted in equal parts with 1 per cent. carbolic acid in normal saline solu-

tion. Samples of serum were then kept in the ice-chest for use as required. For use they were further diluted, if necessary, with saline solution immediately prior to being used in the tests until the required dilution was obtained. A complete list of the serum samples obtained is as follows:—

Serum Sample No.	Source.	Positive or Negative.
1	Natural Infection	Positive.
2	Natural Infection	Positive.
3	Natural Infection	Positive.
4	Natural Infection	Positive.
5	Natural Infection	Positive.
6	Natural Infection	Positive.
7	Institute Cow No. 36	Negative.
8	Natural Infection	Positive.
9	Direct contact with infected animals	Inoculated with virus in the tail and reacted. No C.P.P. on P.M.
10	Direct contact with infected animals	Inoculated with virus in the tail and reacted. No C.P.P. on P.M.
11	Natural Infection	Positive.
12	Natural Infection	Positive.
13	Natural Infection	Positive.
14	Natural Infection	Positive.
15	Natural Infection	Positive.
16	Natural Infection	Positive.
17	Experimental Cow No. 2 (before inoculation)	Negative.
18	Natural Infection	Positive.
19	Institute Cow No. 37	Negative.
20	Direct contact with infected animals	Inoculated with virus in tail and reacted. No C.P.P. on P.M.
21	Direct contact with infected animals	Inoculated with virus in tail and reacted. No C.P.P. on P.M.
22	Direct contact with infected animals	Inoculated with virus in tail and reacted. No C.P.P. on P.M.
23	Natural Infection	Positive.
24	Natural Infection	Positive.
25	Natural Infection	Positive.
26	Natural Infection	Positive.
27	Natural Infection	Positive.
28	Natural Infection	Positive.
29	Experimental Cow No. 4	Reacted to inoculation behind shoulder.
30	Calf No. 1	Immunised by an inoculation in the tail, followed by inoculations behind the shoulder.
31	Institute Cow No. 35	Negative.
32	Calf No. 2	Immunised by an injection in the tail, followed by inoculations behind the shoulder.

Agglutination.

Macroscopic Method.

For the purposes of an agglutination test a culture (second subculture 20 days old) of the organism in Martin's broth plus 7.5 per cent. ox serum was taken and tested with known positive and negative sera, Nos. 8 and 7 respectively. The ingredients were mixed in small agglutination tubes in the following proportions, and placed in the incubator at 37°C. for 24 hours when the result was read.

Positive Serum, No. 8.

Tube.	Culture (undiluted).	Serum (1 in 1).	Carbol* saline.	Result.
	c.c.	c.c.	c.c.	
1	1	1	1	—
2	1	0·75	1·25	—
3	1	0·5	1·5	—
4	1	0·25	1·75	—
5	1	0·1	1·9	—
6	1	0·06	1·94	—
7	1	0·04	1·96	—
8	1	—	2	—
9	—	1	2	—
10	1	—	—	—

Negative Serum, No. 7.

	c.c.	c.c.	c.c.	
1	1	1	1	—
2	1	0 75	1·25	—
3	1	0·5	1·5	—
4	1	0·25	1·75	—
5	1	0·1	1·9	—
6	1	0·06	1·94	—
7	1	0·04	1·96	—
8	1	—	2	—
9	—	1	2	—
10	1	—	—	—

No agglutination was perceptible in any tube of either series, when the result was read after 24 hours' incubation at 37°C. The tubes were allowed to stand at room temperature for a further period of 16 hours, and were again examined, but no different result was obtained at this second reading.

In the conduct of these agglutination tests, those cultures were selected which showed a maximum amount of opalescence. It

* 0·5% carbolic acid in normal saline solution.

should be noted, however, that the opalescence present in any of the cultures was extremely slight, and after the addition of serum and saline to the culture in the test, it was practically impossible to recognise any opalescence at all. In consequence of that fact, there was very little basis for comparing one tube with another, in order to note whether there had been any clearing of the fluid in the tubes.

Even if complete agglutination of all the organisms present in any tube were to take place, it is certain that the resulting deposit of agglutinated micro-organisms would be very slight indeed, and it is possible that it might not be easily recognisable as a deposit, particularly when no information can be obtained from an examination of the fluid for clearing.

An agglutination test was again set up, using as test sera Nos. 3 (positive) and 17 (negative). An alteration was made in the total quantity of the ingredients in the series of tubes, 1.5 c.c.s. of culture being taken as the standard amount of culture for each tube. The other ingredients were added to each tube in their proper quantities, viz., the quantities used in the first test. These tubes were also placed in the incubator at 37°C. for 24 hours, then examined, and then allowed to stand at room temperature for a further period of 16 hours, and again examined. There was no recognisable agglutination in any tube in either series, and altogether the result was exactly similar to that obtained with the test of sera Nos. 7 and 8.

Simultaneously with the test of sera Nos. 3 and 17, another test was set up, the sera used being Nos. 30 and 17. Serum No. 30 was obtained from Calf 1, an experimental animal used at the Veterinary Research Institute, and one which had reacted to a subcutaneous inoculation in the tail of virulent serosity, taken from the lungs of an active case of pleuro-pneumonia. This calf had subsequently received a subcutaneous inoculation of 5 c.c.s. of virulent serosity behind the shoulder without any extensive swelling being produced. Later, it had received 15 c.c.s. of an 8 days' old pure culture of the organism in Martin's broth ox serum subcutaneously behind the other shoulder without any swelling or tissue change being produced at the site of inoculation. It was therefore assumed that Calf 1 had acquired a considerable degree of immunity against the inoculation of virulent pleuro-pneumonic material. For the test with this serum a second subculture 22 days old, in Martin's broth ox serum was

used as the test culture. The result of the agglutination test with this serum was as follows:—

Serum No. 30.

Tube.	Culture (undiluted).	Serum (diluted) (1 in 1).	Carbol Saline.	Result at 24 hours.	Result at 40 hours.
	c.c.	c.c.	c.c.		
1	1·5	1.0	1·0	+	++
2	,,	0·75	1·25	+	++
3	,,	0·5	1·5	?	+
4	,,	0·25	1·75	—	?
5	,,	0·1	1·9	—	—
6	,,	0·06	1·94	—	—
7	,,	0·04	1·96	—	—
8	,,	—	2	—	—
9	—	1	2·5	—	—
10	1·5	—	—	—	—

Serum No. 17.

	c.c.	c.c.	c.c.		
1	1·5	1	1	—	—
2	,,	0·75	0·25	—	—
3	,,	0·5	1·5	—	—
4	,,	0·25	1·75	—	—
5	,,	0·1	1·9	—	—
6	,,	0·06	1·94	—	—
7	,,	0·04	1·96	—	—
8	,,	—	2	—	—
9	—	1	2·5	—	—

++ = Agglutination and sedimentation. — = Negative reaction.
+ = Recognisable agglutination. ? = Doubtful reaction.

In tubes 1 and 2 in the Serum-No.-30 series, agglutination was perceptible after 24 hours' incubation at 37°C. The amount of deposit in either tube was very small, but it could be clearly recognised. Tube 3, after 24 hours' incubation, appeared to show some agglutination, but it was so indefinite it could not be recorded as a "positive" result. When read after standing at room temperature for a further period of 16 hours, tube 3 showed agglutination, while tube 4 showed an indefinite agglutination similar to tube 3 at 24 hours. Tubes 1 and 2, after standing at room temperature showed agglutination and sedimentation, which was very clearly recognisable. The reactions with Serum No. 17 were negative throughout, while all the controls were negative also.

Having obtained a positive agglutination reaction with the serum of an animal which had been experimentally inoculated, the

question naturally arises: How can the failures to secure positive reactions with the sera from animals which are naturally affected with the disease be explained? It is conceivable, of course, that the amount of antibody (agglutinin) substance present in the blood serum of a naturally infected animal falls far short of the amount which is produced in an experimental animal as the result of several subcutaneous injections of virulent serosity and culture, and that, while with the latter serum agglutination can be observed as a macroscopic reaction, with the serum of a naturally infected animal agglutination may be only partially complete, and may not be recognisable macroscopically, if it takes place at all.

An important point which must not be overlooked is the method adopted in the immunisation of Experimental Calf 1. This animal was injected subcutaneously with virulent serosity, and subsequently with virulent serosity, and later with pure culture in Martin's broth ox serum.

The serum of an animal immunised in such a manner might possibly contain specific precipitins for ox serum, in which case turbidity in the reaction obtained with the serum of Calf 1, when tested for agglutinins, may have been due to some precipitin element, at least in part.

Zinsser (1914) (21), in discussing the specificity of precipitins and the precipitin reaction, refers to the experiments of Uhlenhuth, who "obtained a specific anti-hare serum by treating rabbit's blood with hare's blood—an astonishing result, in view of the close zoological relations between these animals. Isoprecipitins, that is, precipitins resulting from the treatment of animals with blood from another individual of the same species, have also been described by Schutze and others. They are not, however, regular in their appearance, nor are they very potent when obtained."[11]

It is very improbable, therefore, that an isoprecipitin would be formed in the blood of Calf 1, as a result of the immunising injections of virulent serosity and culture in Martin's broth, each of which injections contained a small amount of ox serum. Even assuming that an isoprecipitin was present, it scarcely seems possible that the serum of Calf 1 could produce by a precipitin reaction alone, such a definite deposit as that which occurred in the agglutination test already referred to.

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Microscopic Method.

In order to test whether the usual microscopic method of observing the agglutination reaction could furnish any additional evidence over the macroscopic method, two hanging-drop preparations were made under cover slips on hollow glass slides, the cover slips being ringed round with vaseline in order to prevent evaporation of the hanging drops. One hanging drop consisted only of pure culture of the organism in Martin's broth plus ox serum, and on microscopic examination showed no trace of any recognisable micro-organisms when using the highest magnification available (1000 diameters). The other hanging drop consisted of a mixture of the same culture, and a positive serum in the proportion of 2 parts of culture to 1 part of serum dilution, serum No. 12 being used. This preparation was examined microscopically at various intervals extending over 4 hours, but at no time could any alteration in the appearance of the hanging drop be observed. No difference in appearance under the microscope could be noted when the hanging drop containing culture and serum was compared with that containing culture only.

In order to have a further means of comparison it was decided to repeat this hanging drop test, and to control it more efficiently, two additional hanging drop preparations were put up. Thus we had for examination four hanging drop preparations containing the following ingredients respectively:—

Hanging-Drop Preparation No. 1, containing culture only.

Hanging-Drop Preparation No. 2, containing culture (2 parts), and serum dilution (1 part); the serum being Serum No. 12 (positive serum).

Hanging-Drop Preparation No. 3, containing culture (2 parts), and serum dilution (1 part); the serum being Serum No. 30.

Hanging-Drop Preparation No. 4, containing culture (2 parts), and serum dilution (1 part); the serum being No. 17 (negative serum).

Serum No. 30 was the serum from Experimental Calf 1, which had previously given a positive agglutination reaction when tested by the macroscopic method.

Serum No. 17 was a negative serum, and had previously given a negative agglutination reaction when tested by the macroscopic method. The preparations were examined microscopically at

intervals extending over 5 hours, during which time no alteration was apparent in any of the preparations. Even in Preparation No. 3, which contained Serum No. 30, no agglutination could be recognised under the microscope.

Agglutination Experiments with Concentrated Culture.

In conducting the macroscopic agglutination test it had been found that the dilution of culture brought about by the addition of test serum made it impossible to recognise any opalescence in the fluid in the tubes. It was therefore considered advisable to attempt to produce a concentration of the culture so that the opalescence would be more distinct, and would be clearly visible when the other ingredients of the test were added. Culture in Martin's broth ox serum was placed in centrifuge tubes and whirled in an electric centrifuge at the highest speed the machine was capable of attaining (2500 revolutions per minute) for 4½ hours. At the end of that time the tubes were examined, but it was found that it was not possible to effect concentration of micro-organisms in that manner, because the opalescent particles (organisms) in the broth were so fine and light they could not be thrown down to the bottom of the tubes.

An attempt was then made to concentrate culture by the evaporation of some of the fluid medium in which the culture was growing. This evaporation was first tried at room temperature by placing a quantity of culture in a flat dish (Petri dish) inside a desiccator, provided with a circular trough, which contained pure sulphuric acid. Inside the desiccator a more or less complete vacuum was established and maintained. It was found that with such an apparatus, the rate of evaporation at room temperature was too slow, so the apparatus was placed in the incubator and evaporation attempted at incubator temperature. At incubator temperature, and with the joints of the desiccator sealed with "plasticine" (the temperature made it impossible to use vaseline for the purpose), there was difficulty in maintaining the vacuum, and, altogether, the experiment was not a success. It was then decided to attempt the evaporation of culture at room temperature by using a method, of which the following is a description:—

424 c.c. of culture in Martin's broth plus ox serum was placed in a flask (Flask A), furnished with a side-arm, and the mouth of the flask was firmly closed with a tightly fitting solid rubber

stopper. A length of india-rubber pressure tubing was attached to the side arm of Flask A, the other end of the tubing being attached to the side arm of Flask B. Flask B was empty, and was closed at the mouth by an india-rubber stopper, which was perforated to give passage to a length of glass tubing, one end of which extended to the bottom of Flask B. On the other end of this glass tube another piece of india-rubber pressure tubing was attached, and was connected to a piece of glass tubing perforating the india-rubber stopper of Flask C, which was a flask exactly similar to Flask B. A piece of india-rubber pressure tubing connected Flask C by the side arm to the glass tubing perforating the cork of a Sulphuric Acid Tower (D). The sulphuric acid tower was connected by rubber tubing to an aspirator. (A Korting pattern water pump.) This aspirator maintained a negative pressure in Flasks A, B, C, and in the tower D. Flask A, containing the culture was kept at room temperature. Flask B was placed in a receptacle containing crushed ice and salt, the flask being quite covered by the freezing mixture. Additional ice and salt were added from time to time as required. The fluid, which collected in Flask B, was transferred to Flask C by aspiration. At the end of 24 hours the apparatus was dismantled, and the contents of Flask A were measured, and were found to be only 173 c.c.s.; 251 c.c.s. of clear watery solution having passed over into Flasks B and C. This 173 c.c.s. of concentrated culture showed a very marked turbidity, and a quantity of it was used as an antigen (Antigen J) in the complement fixation test, and a further quantity was used for agglutination tests as follows:—

First Test.

Concentrated culture with Serum No. 26 (positive), and Serum No. 7 (negative).

Serum No. 26 (Positive).

Tube.	Culture (concentrated).	Serum (diluted 1 in 1).	Carbol Saline.	Result at 24 hours.	Result at 40 hours.
	c.c	c.c.	c.c.		
1	1·5	1	1	—	—
2	,,	0·75	1·25	—	—
3	,,	0·5	1·5	—	—
4	,,	0·25	1·75	—	—
5	,,	0·1	1·9	—	—
6	,,	0·06	1·94	—	—
7	,,	0·04	1·96	—	—
8	,,	—	2	—	—
9	—	1	2.5	—	—
10	1·5	—	—	—	—

SERUM No. 7 (NEGATIVE).

		c.c.		c.c.		c.c.				
1	-	1·5	-	1	-	1	-	—	-	—
2	-	,,	-	0·75	-	1·25	-	—	-	—
3	-	,,	-	0·5	-	1·5	-	—	-	—
4	-	,,	-	0·25	-	1·75	-	—	-	—
5	-	,,	-	0·1	-	1·9	-	—	-	—
6	-	,,	-	0·06	-	1·94	-	—	-	—
7	-	,,	-	0·04	-	1·96	-	—	-	—
8	-	,,	-	—	-	2	-	—	-	—
9	-	—	-	1	-	2·5	-	—	-	—
10	-	1·5	-	—	-	—	-	—	-	—

— = Negative reaction.

Second Test.

Concentrated culture with Serum No. 23 (positive), and Serum No. 31 (negative).

This test was set up in exactly the same manner as the preceding test (Test 1), and the result obtained was exactly similar to that obtained with Test 1.

Third Test.

Concentrated culture with Serum No. 30 (from Experimental Calf 1), and Serum No. 31 (negative).

SERUM No. 30 (POSITIVE).

Tube.	Culture (concentrated).		Serum (diluted 1 in 1).		Carbol Saline.		Result at 24 hours.		Result at 40 hours.
	c.c.		c.c.		c.c.				
1	- 1·5	-	1	-	1	-	++	-	++
2	- 1·5	-	0·75	-	1·25	-	++	-	++
3	- ,,	-	0·5	-	1·5	-	++	-	++
4	- ,,	-	0·25	-	1·75	-	+	-	+
5	- ,,	-	0·1	-	1·9	-	+	-	+
6	- ,,	-	0·06	-	1·94	-	?	-	?
7	- ,,	-	0·04	-	1·96	-	—	-	—
8	- ,,	-	—	-	2	-	—	-	—
9	- —	-	1	-	2·5	-	—	-	—
10	- 1·5	-	—	-	—	-	—	-	—

SERUM No. 31 (NEGATIVE).

		c.c.		c.c.		c.c.				
1	-	1·5	-	1	-	1	-	—	-	—
2	-	,,	-	0·75	-	1·25	-	—	-	—
3	-	,,	-	0·5	-	1·5	-	—	-	—
4	-	,,	-	0·25	-	1·75	-	—	-	—
5	-	,,	-	0·1	-	1·9	-	—	-	—
6	-	,,	-	0·06	-	1·94	-	—	-	—
7	-	,,	-	0·01	-	1.96	-	—	-	—
8	-	,,	-	—	-	2	-	—	-	—
9	-	—	-	1	-	2·5	-	—	-	—
10	-	1·5	-	—	-	—	-	—	-	—

++ = Agglutination and sedimentation. — = Negative reaction.
+ = Agglutination. ? = Doubtful Reaction.

The marked turbidity of the concentrated culture made it possible to still recognise some turbidity in the tubes when the necessary amounts of serum and saline were added, so that, in these tests with concentrated culture, it was possible to compare the fluid in the tubes for any clearing which might take place during the test. Excepting with the tubes containing Serum No. 30, no clearing of the test fluid occured, and no agglutination could be demonstrated when serum from a naturally infected animal was mixed with a culture of the filter passing organism isolated from a case of contagious pleuro-pneumonia.

The failure to obtain a recognisable agglutination reaction with this concentrated culture when mixed with the sera of naturally infected animals made us abandon the agglutination test as a potential diagnostic reaction for the detection of contagious pleuro-pneumonia in the living animal.

Ability of the Organism to Grow in Media containing Immune Sera.

On the conclusion of these aggultination tests it was decided to try if the addition to the culture medium of serum from naturally infected and from experimentally inoculated animals would influence the growth of the organisms when subcultures were made into such media. For this experiment, three separate batches of media were prepared. The first consisted of Martin's Peptone Broth plus 7.5 per cent. of Serum No. 27, which was obtained from a naturally infected animal. The second consisted of Martin's Peptone Broth, plus 7.5 per cent. of serum No. 30, which was obtained from Experimental Calf 1. The third consisted of Martin's Peptone Broth plus 7.5 per cent. of normal ox serum.

Several tubes of each batch of medium were inoculated from a primary culture of the organism in Martin's broth plus normal ox serum. Growths took place in all the tubes inoculated. In the tubes containing Serum No. 27 (from a naturally infected animal) the characteristic opalescence was observed four days after inoculation and incubation at 37°C., and on comparison with the tubes of Martin's broth, plus normal ox serum, inoculated at the same time from the same source, no difference in the degree of opalescence, or in the general appearance of the cultures could be observed. On comparing the cultures containing the serum from Experimental Calf 1 with those containing Serum No. 27,

it was distinctly noticeable that in the former tubes the characteristic opalescence was more marked, but there was no recognisable sedimentation at the bottom of the culture tubes. It is not considered that the increased opalescence in the cultures growing in the media containing the serum of Experimental Calf I was produced by a more vigorous growth of the organisms in those tubes, but in all probability it was due to a partial agglutination of the organisms in the culture fluid, and the interception of rays of light in their passage through the culture fluid by the slight agglomerations of partly agglutinated organisms floating in the culture media. The experiment was adequately controlled by incubating at the same time tubes of the same media which had not been inoculated with the primary culture. These control tubes maintained an unaltered appearance throughout.

Complement Fixation.

To test the complement fixation reaction in contagious pleuro-pneumonia, the following ingredients for the test were prepared as required.

(1) Haemolytic Amboceptor.—The serum of a rabbit immunised by repeated injections of the washed red blood corpuscles of a sheep. This rabbit's serum was inactivated by heating in a water bath at 56°C. for 30 minutes, and for the tests was employed in a dilution of 1 in 1000 with saline solution. The exact dilution and the amount for the test was established at the commencement of the test by one or more titration experiments. The titre of this Haemolytic Amboceptor was never less than 1 in 1000 when used in any of the complement fixation tests.

(2) Corpuscle Suspension.—A 5 per cent. suspension in saline solution of the washed red blood cells of a sheep.

(3) Complement.—Fresh guinea-pig's serum, diluted 1 in 10 with saline solution, the minimum haemolytic dose (M.H.D.) being established by titration.

(4) Saline Solution.—0.9 per cent. sodium chloride solution, filtered, and sterilised.

(5) Test Sera.—Obtained from Bovines.

In the earlier complement fixation tests, which were carried on over a period of several months, the test sera were diluted with equal parts of 1 per cent. carbolic acid in saline solution for preservation, and were kept in the ice chest for use as required. They were further diluted with physiological saline solu-

tion immediately prior to use in the tests. Although the quantity of carbolic acid present in the amount of serum dilution used in the tests was infinitesimal, it was considered that better results might be obtained if the carbolic acid was eliminated altogether. It was therefore decided to inactivate the bovine sera at 56°C. for half an hour on two consecutive days as soon as possible after collection, and store them in the ice chest in an undiluted form for use as required. It has been found that sera so treated invariably remain clear and sterile.

Tests made with two quantities of serum from the one animal, one quantity of which was only once heated at 56°C. for half an hour, the other quantity being heated at 56°C. for half an hour on two consecutive days, showed no difference in their complement fixing properties. The double heating is advisable if the sera are to be preserved for any length of time.

(6) Antigen.—Cultures in Martin's broth plus ox serum or horse serum were used as Antigens, but before use were prepared as follows:—The culture was heated in a water bath at 56°C. for one hour, then diluted in equal parts with 1 per cent. carbol saline solution. The mixture was then placed in a mechanical shaking apparatus, and thoroughly shaken for 5 hours. It was then placed in the ice chest for 24 hours, and before use in the test was further diluted by using 1 part of culture mixture to 4 parts of 0.9 per cent. saline solution, forming a 1 in 10 dilution of the original culture.

With the exception of "Antigen A," all the culture preparations used from time to time as antigens in the complement fixation tests were prepared in the above manner. The various cultures prepared and tested as antigens are enumerated in the following table:—

Antigen A.—A fourth subculture of the filter-passing organism in Martin's broth ox serum. It was first used on 18/11/19, after 8 days of growth, in a 1 in 10 dilution with saline solution. It was anticomplementary when any quantity in excess of 0.1 c.c. of a 1 in 10 dilution was used. On 24/11/19, it was not anticomplementary when used in an amount of 0.25 c.c. of a 1 in 10 dilution. On 11/12/19 (after 30 days of growth) it showed anticomplementary action in quantities of 0.1 c.c. of a 1 in 10 dilution in saline.

Antigen B.—A first subculture, 12 days old, in Martin's broth ox serum. Not anticomplementary in doses of 0.25 c.c. of a 1 in 10 dilution.

Antigen C.—A third subculture, 14 days old, in Martin's broth-horse serum. (Primary culture in Martin's broth ox serum, first and second subcultures in Martin's broth horse serum.) It was markedly anticomplementary in quantities of 0.25 c.c. of a 1 in 10 dilution, but gave no inhibition of haemolysis in quantities of 0.15 c.c. of a 1 in 10 dilution.

Antigen D.—A divalent antigen. A mixture of a third subculture 8 days old, and a second subculture 10 days old, from two separate sources. Both cultures were grown in Martin's broth plus ox serum. Not anticomplementary in quantities of 0.25 c.c. of a 1 in 10 dilution.

Antigen E.—A 28 days old culture (filtration experiment) in Martin's broth ox serum. Not anticomplementary in quantities of 0.2 c.c. of a 1 in 10 dilution. Slight inhibition with 0.25 c.c. of the same dilution.

Antigen F.—Lung serosity from an active case of contagious pleuro-pneumonia. Very anticomplementary in all quantities of a 1 in 10 dilution. 0.5 c.c. of a 1 in 20 dilution in saline showed no inhibition of haemolysis.

Antigen G.—Oedematous fluid from the zone of inoculation behind shoulder (Experimental Cow 4), taken 16 days after inoculation. Not anticomplementary in quantities of 0.3 c.c. of a 1 in 10 dilution.

Antigen H.—A polyvalent antigen made up of four strains of culture in Martin's broth ox serum. (One culture 8 days old; one culture 14 days old; one culture 16 days old; and one culture 21 days old). Culture mixture not anticomplementary in quantities of 0.25 c.c. of a 1 in 10 dilution.

Antigen J.—Concentrated culture in Martin's broth plus ox serum, concentrated by evaporation (*vide supra*).

Complement Fixation Tests with Pure Culture.

These various culture preparations were tested for complement fixation with known positive and negative sera. A number of tests were conducted with each antigen preparation, but in every case the test showed that the antigen plus positive serum combination did not fix complement; the final result in all such cases being complete haemolysis Antigen plus negative serum gave complete haemolysis also; thus, complete haemolysis took place irrespective of whether the serum used was obtained from a positive or negative source.

Serum No. 30 from Experimental Calf 1 (the animal whose serum had previously given a positive agglutination reaction when tested by the macroscopic method) was tested with various culture preparations including Antigen J. There was no fixation of complement in any combination tested, and the haemolysis was just as complete in the positive serum series as it was in the negative serum series put up at the same time.

Culture preparations (antigens) obtained by the cultivation of virulent serosity from the lungs of the animal supplying positive Serum No. 28, were tested with this latter serum for complement fixation. It was found that, in each instance, the reactions were negative.

Various other tests were carried out with culture preparations which it is unnecessary to enumerate. Suffice it to say that in no case, using culture as an antigen, was a positive complement fixation result obtained.

Discussion.

At this stage it was decided to review that portion of the work relating to complement fixation, in an endeavour to ascertain the reason why, in the tests carried out, it had been impossible to obtain complement fixation with a known positive serum.

It is conceivable, of course, that filterable viruses in general do not behave in the same way as bacteria, i.e., they may not react to the usual serological tests that bacteria react to. This idea is strengthened by the fact that, although the usual serological reactions have been tested with a number of other filterable viruses, as well as with contagious pleuro-pneumonia by several experienced workers, the results obtained have either been negative, or else so contradictory that it has been impossible to elaborate a test, using the ordinary technique of such serological reactions, which could be used with certainty for diagnostic purposes. On the other hand it is possible that certain serological reactions can be obtained, provided a special technique is employed, either in the preparation of the component parts of the test, or in the method of conducting the test, or both.

Antigens.

The first question which presented itself on reviewing the earlier complement fixation experiments was: Were the antigens used in those experiments strong enough in a specific sense to bring about complement fixation? It will have been noted

that when antigens consisting of pure cultures of the organism of contagious pleuro-pneumonia in Martin's broth were used, complement fixation did not occur.

These results were capable of one of two interpretations:—

(1) That culture was unsuitable for antigenic purposes;

(2) That complement fixing antibodies were not present in the serum of animals affected with contagious pleuro-pneumonia.

The first interpretation was temporarily accepted as the more probable. The second could not be accepted without further evidence; so, in order to determine whether complement fixing antibodies were or were not present in the serum of animals affected with contagious pleuro-pneumonia, tissue extracts were next prepared and tested as antigens.

The first tissue extracts prepared were saline extracts of diseased lung tissue, taken from animals showing active and extensive lesions of contagious pleuro-pneumonia on post-mortem examination. These saline extracts of diseased lung tissue were prepared as follows:—

Carefully selected diseased lung tissue was cut into small pieces and ground up in a mortar with a little sterile sea sand. Four times its weight of carbol saline solution was added, and the mixture placed in a tightly stoppered bottle, and shaken for five hours in a mechanical shaking apparatus. The mixture was then filtered through gauze to remove all the coarser particles of the tissue. It was then placed in the ice chest for a week, to allow sedimentation to take place, after which time the supernatent fluid was carefully pipetted off without disturbing the deposit. The supernatent fluid—which is yellowish-brown in colour, and slightly opalescent—was then diluted 1 part in 10 with 0.9 per cent. saline solution, and was used in that dilution in the test, after titration to determine the anticomplementary unit.

These saline extracts were thoroughly tested with a number of known positive and known negative sera, but in no instance was a positive fixation result obtained.

A saline extract of the sub-epidermal tumour tissue removed from the inflammatory tumour, which had developed behind the shoulder of an experimentally inoculated animal (Experimental Cow 4) was next prepared. This tissue was not regarded as entirely satisfactory for test purposes, for, at the time of its removal, the tissue was showing marked evidence of necrosis. The method adopted in the preparation of this tissue extract differed

slightly from the method employed for the preparation of the saline extracts of lung tissue, and was as follows:—10 grams of the inflamed subepidermal tumour tissue was cut into small pieces, and ground up in a mortar with a little sterile sea sand. 40 c.c.s. of 0.5 per cent. carbolic acid in 0.9 per cent. saline solution was then added, and the mixture placed in a tightly stoppered bottle, and shaken in a mechanical shaker for five hours. It was then filtered through gauze to remove the coarser particles, and then placed in the ice chest for a week, being shaken up occasionally. It was finally allowed to sediment, and the supernatent fluid decanted. This fluid was filtered through filter paper, and again placed in the ice chest for a further 48 hours. The supernatent fluid was then carefully pipetted off without disturbing the slight deposit in the bottle. 1 c.c. of this fluid was then diluted with 9 c.c.s. of 0.9 per cent. saline solution, and was titrated to determine the anticomplementary unit. This 1 in 10 dilution was used in the test.

When tested with positive sera Nos. 11 and 25, this saline extract gave a negative reaction, but when tested with Serum No. 30 (from Experimental Calf 1), a positive complement fixation was recorded. This was the first positive complement fixation result recorded, although Serum No. 30 had been tested with practically every antigen preparation which had been previously tried.

It should be pointed out that Experimental Calf No. 1 had been specially immunised by several subcutaneous inoculations of virulent material; therefore, the serum of Calf 1 would be expected to be highly specific, and not properly comparable with the serum of an animal affected with the disease naturally acquired.

As extracts of sub-epidermal tumour tissue apparently offered the most favourable chances of obtaining a successfully reacting antigen, a number of saline extracts were prepared from subepidermal tumour tissue, taken from behind the shoulder of Experimental Cow 6 (who was destroyed owing to the extensive infiltrating oedema which had developed as a result of the inoculation of pure virus behind the shoulder, and from which the animal was dying). This extract gave a strongly positive result when tested with Serum No. 30, and gave some fixation with positive sera Nos. 33, 36, 37 and 39, but with positive sera Nos. 34, 35, 35a, 35b, and 38, the results were negative; haemolysis taking place in the antigen containing tubes just as readily as in the serum control tubes which did not contain antigen.

Alcoholic Extracts of Diseased Tissue used as Antigen.

Saline extracts of diseased tissue having proved unsatisfactory for antigen purposes, alcoholic extracts were next prepared and tested. Thin slices of inflamed sub-epidermal tumour tissue taken from Cow 6, when the animal was destroyed, had been dried in the incubator at 37°C. for some days, and preserved in the dry state in a closely stoppered bottle. Of this dried tissue, 5 grams were taken, and finely pulverised in a mortar with the addition of a small quantity of powdered glass. 50 c.c.s. of alcohol were then added, the whole placed in a tightly stoppered bottle, and placed in a mechanical shaking apparatus and thoroughly shaken for 12 hours. It was then stored in the ice chest for 12 days, being shaken up at least once a day. The fluid was then poured off into centrifuge tubes, and whirled in the centrifuge. The supernatent fluid was then carefully pipetted off without disturbing the deposit. 1 c.c. of this alcoholic extract was diluted with 9 c.c.s. of 0.9 per cent. saline solution, the dilution being made slowly in order to obtain the maximum amount of turbidity. This antigen dilution was then titrated in order to ascertain its anticomplementary and complementary units.

An important point of technique in the preparation of this antigen for test purposes is the method of diluting the alcoholic extract with saline solution.

If the alcoholic extract and saline solution are mixed quickly a slightly turbid mixture results which gives only slight fixation when tested with known positive sera. On the other hand, when the alcoholic extract and saline solution are mixed slowly, an extremely turbid mixture is obtained, the amount of turbidity being in direct proportion to the time allowed for mixing. The mixtures possessing the maximum amount of turbidity have been found on testing to give the maximum amount of fixation.

The alcoholic extract of sub-epidermal tumour tissue was tested with a number of known positive and known negative sera, and after having been tested with 24 different sera, it was found that the test result in each case was in agreement with the post-mortem findings when the animals supplying the test sera were slaughtered and examined.

Thus it is evident that complement fixing antibodies are present in the sera of animals affected with contagious pleuro-pneumonia, and the complement fixation test provides us with a means of differentiating between infected and non-infected animals.

Conglutinin.

It was found, when carrying out tests with the alcoholic extract of sub-epidermal tumour tissue, that, while complement fixation occurred with known positive sera, the reaction was liable to become masked by a subsequent haemolysis of the sensitised red blood cells added as an indicator.

Different positive sera exhibited this tendency to bring about final haemolysis in varying degrees—i.e., some were more prone to it than others—but as haemolysis invariably occurred, it was evident that there was contained in the test serum some element which was capable of acting upon the sensitised red blood cells after fixation of complement had occurred. This haemolysis made it exceedingly difficult to differentiate between the reaction given by a known negative serum, and a known positive serum, because, in some cases, the length of time between the haemolysis with a negative serum, and the haemolysis with a positive serum was only a matter of a few minutes.

It was also noticed that in the final test, on the addition of the sensitised red blood cells, the red blood cells were almost immediately "agglutinated" and sedimented more or less completely at the bottom of the tubes.

It was thought at first that this "agglutination" of red blood cells could be made use of for diagnostic purposes, but it was very soon demonstrated that, while, as a general rule, the phenomenon occurred earliest in the tubes containing negative sera, certain of the tubes containing positive sera also showed early "agglutination," of the red blood cells.

On the other hand, some tubes containing negative sera showed a delayed "agglutination" of the red blood cells. A similar delayed "agglutination" was also shown in certain of the tubes containing positive sera.

It was thus evident that this phenomenon was of no diagnostic importance, because its appearance depended upon some substance which was present in all the bovine sera tested, although present in a varying amount. This substance was not more often present in negative sera than in positive sera, although, as a general rule, the reaction appeared earlier with negative sera.

Bordet and Gay (1906) (3) in studying the action of inactivated bovine serum upon sensitised corpuscles in the presence of complement, demonstrated the presence in bovine serum of a specific substance which they first referred to as "colloid sub-

stance," but which they afterwards termed "conglutinin." The name "conglutinin" was suggested by the action of this substance upon sensitised red blood cells, with a suitable complement. In such a combination the red blood cells were energetically "agglutinated" and sedimented. Although the reaction was similar to the action of a powerful agglutinin, it differed from the action of an agglutinin in that complement was a necessary ingredient in the combination for this phenomenon to occur. Complement is not necessary to complete the action of an agglutinin.

These authors found that this substance—conglutinin—present in varying amounts in bovine sera, was capable of exercising a remarkable influence upon the final result in a haemolytic test. In a series of experiments they demonstrated that the substance, acting in conjunction with a weak complement, could conglutinate and haemolyse sensitised red blood cells, although the amount of complement taking part in the reaction, by itself and without conglutinin, was incapable of producing haemolysis. In other words, it was demonstrated that conglutinin could combine with a weak complement, and the combination could then exert a powerful haemolytic action upon sensitised red blood cells.

In the technique of the complement fixation tests described in the previous chapter, the complement was titrated against one unit of sensitised red blood cell suspension, in order to determine the minimum haemolytic dose (M.H.D.) of complement. Against this standardised haemolytic system the other ingredients of the test were titrated, in order to ascertain the proper quantities of each to employ in the final stage of the test. When, however, in the final test, bovine serum and complement are mixed together, and incubated, and then sensitised red blood cells are added, the influence of conglutinin becomes exerted, and the previously titrated M.H.D. of complement apparently becomes more than the M.H.D. required for haemolysis, so that a fixation with a positive serum may still leave sufficient complement available to be reinforced by the conglutinin present, and produce haemolysis of the sensitised red blood cells.

In order to test this hypothesis, the following experiment was carried out:—

A series of tubes (Series A) was set up, and into each tube was measured decreasing quantities of fresh guinea-pig's serum (1 in 10 dilution), from 0.5 c.c. downwards. The fluid in all

the tubes was then brought to a common level by the addition of 0.9 per cent. saline solution, so that each tube contained 2.5 c.c. of fluid. One unit of sensitised red blood cells (sheep) was then added to each tube, and, after shaking, the tubes were placed in the incubator at 37°C. Results were read at the end of half an hour, one hour, and two hours' incubation respectively, and, of the tubes showing complete haemolysis, the one containing the smallest amount of complement was noted. From the table set out below it will be seen that this tube was tube 6, which contained 0.25 c.c. of complement dilution; that amount being the minimum quantity required to completely haemolyse one unit of sensitised red blood cells in one hour at 37°C. Even after two hours' incubation it was found that no tube lower than tube 6 in the series showed complete haemolysis.

Parallel with series A, series B was set up. The quantities of sensitised red blood cells and complement dilution were exactly the same as those used in the corresponding tubes in series A, but to each tube in series B (excepting where indicated in the controls), 0.15 c.c. of a 1 in 10 dilution of inactivated bovine serum was added. The tubes were filled to a common level with saline solution, as in series A, and placed in the incubator at 37°C., the results being read after half an hour, one hour, and two hours' incubation. Each series was thoroughly controlled, as shown in the following tables:—

Series A.

Tube	Complement, 1 in 10	Saline 0.9%	Sensitised R.B.C. 5%	Degree of Haemolysis after incubation for		
				Half hour	One hour	Two hours
1	·5	2·0	·5	Complete	Complete	Complete
2	·45	2·05	·5	,,	,,	,,
3	·4	2·1	·5	,,	,,	,,
4	·35	2·15	·5	,,	,,	,,
5	·3	2·2	·5	,,	,,	,,
6	·25	2·25	·5	Almost complete	Complete	Complete
7	·2	2·3	·5	Partial	Partial	Not complete
8	·15	2·35	·5	Very slight	Slight	Slight
9	·1	2·4	·5	None	Very slight	Slight
10	·05	2·45	·5	None	None	None
11	—	2·5	·5	None	None	None

SERIES B.

Tube.	Inactivated Bovine serum, 1 in 10.	Complement, 1 in 10.	Saline, 0·9%.	Sensitised R.B.C. 5%.	Degree of Haemolysis after incubation at 37°C. for		
					Half hour.	One hour.	Two hours.
1	·15	·5	1·85	·5	Complete	Complete	Complete
2	·15	·45	1·9	·5	„	„	„
3	·15	·4	1·95	·5	„	„	„
4	·15	·35	2·	·5	„	„	„
5	·15	·3	2·05	·5	„	„	„
6	·15	·25	2·1	·5	Partial	„	„
7	·15	·2	2·15	·5	Slight	„	„
8	·15	·15	2·2	·5	Slight	Partial	„
9	·15	·1	2·25	·5	Very slight	Slight	„
10	·15	·05	2 3	·5	None	Slight	Almost complete
11	·15	—	2·35	·5	None	None	None
12	—	—	2·5	·5	None	None	None

The results in series B, read after incubation for half an hour, were almost exactly similar to the haemolytic results obtained with series A, after incubation for half an hour. In series B, however, the sensitised red blood cells were energetically conglutinated shortly after they were added. After incubation for one hour, series B showed complete haemolysis in tubes 1 to 7, and varying degrees of haemolysis in tubes 8, 9, and 10. At the end of two hours' incubation haemolysis was complete in tubes 1 to 9, while in tube 10 haemolysis was almost complete.

It is thus evident that the influence of conglutinin upon a small amount of complement is such that in the complement fixation test for contagious pleuro-pneumonia, it enables haemolysis to occur with a smaller quantity of complement than the ordinarily titrated M.H.D.

If we critically examine the figures revealed by the foregoing experiment, it is at once apparent that the presence of conglutinin in the bovine test serum introduces a factor into the complement fixation test in contagious pleuro-pneumonia, which factor, if not properly controlled, would render the test absolutely unreliable for diagnostic purposes.

It has been shown in this experiment that it requires 0.25 c.c. of complement dilution to completely haemolyse one unit of sen-

sitised red blood cells. The M.H.D. of complement is thus 0.25 c.c. of a 1 in 10 dilution. If now we set up a test as follows:—

Antigen 1 in 10.	Inactivated Bovine serum, 1 in 10.	Complement 1 in 10.	Saline 0·9%	Sensitised R.B.C.5%.
c.c. ·15	c.c. ·15	c.c. ·25	Q.S. to make up to 2.5 c.c.	1 unit

and assume that the bovine serum being tested is a positive serum, we would find that complement would be fixed by the combination of antigen—positive serum; but—and this is the important point—apparently not all the complement is fixed. A certain amount of complement remains unfixed, because it appears that the combination of antigen and antibody in contagious pleuro-pneumonia is only capable of fixing a small amount of complement. The small amount of complement remaining unfixed is less than the M.H.D. required to produce haemolysis of the haemolytic system, but it is reinforced by the conglutinin present in the bovine serum, and thus reinforced, it conglutinates and ultimately haemolyses more or less completely the unit of sensitised red blood cells added to the test as an indicator.

In order to overcome errors due to this action of conglutinin upon a fraction of the M.H.D. of complement, the final reading of the complement fixation test in contagious pleuro-pneumonia has to be made in strict conjunction with an adequate number of control tubes. These will be fully considered later in the section, dealing with "Technique."

In this discussion upon the action of conglutinin, there is another point of some importance, which must be referred to briefly.

It is probable that conglutinin can bring about a conglutination reaction, in the presence of complement, with other antigen-antibody combinations besides the antigen-antibody combination contained in the haemolytic system. For instance, the conglutinin in the bovine serum may vary the reaction of our antigen and antibody (where a positive serum is being used, and especially where culture is used as antigen). If such is the case, the joining up of the antigen-antibody-complement combination in the first portion of the final test for complement fixation may be expected to be delayed considerably, but thereafter sufficient complement may remain free on the addition of the sensitised red blood cells to bring about haemolysis. If this is so, the frequency with which negative complement fixation reactions were obtained in our

earlier experiments with pure culture as antigen, and with sera obtained from known positive cases of contagious pleuro-pneumonia could be explained.

In concluding this section on conglutinin it is perhaps worthy of mention that amongst the many experiments carried out with conglutinin was an experiment to determine whether a modified conglutination reaction, possessed any diagnostic value in contagious pleuro-pneumonia. For this experiment, culture and a known positive serum were mixed together in graded doses, and allowed to remain in the incubator for 12 hours. At the end of that time complement was added to each tube. It was thought that the combination of culture (antigen) and positive serum (antibody) would be conglutinated by the combined action of conglutinin and complement.

Although encouraging results were obtained at first, it was soon demonstrated that this method was quite unreliable for diagnostic purposes.

Quantitative Relationship between Complement and Amboceptor in the Production of Haemolysis.

Morgenroth and Sachs (1902) (11) have demonstrated that within certain limits there exists an inverse relationship between haemolytic amboceptor and complement in the production of haemolysis. If for a given quantity of red blood cells a certain quantity of haemolytic amboceptor and complement is required to bring about complete haemolysis, reduction of either the complement or amboceptor necessitates an increase of the other factor.

Noguchi (1911) (14) has shown that in the presence of one unit of haemolytic amboceptor, 0.1 c.c. of guinea-pig's complement is required to produce complete haemolysis of a given quantity of red blood cells in a given time, while, by using 4, 8, and 20 units of amboceptor, complete haemolysis of a similar quantity of red blood cells is obtainable in the same time with 1/3, 1/5, and 1/10 of the 0.1 c.c. of complement respectively.

This inverse relationship between amboceptor and complement is of the utmost importance in complement fixation work in contagious pleuro-pneumonia, because it enables us to overcome the action of conglutinin to a certain extent.

If in the standardisation of the haemolytic system for complement fixation in contagious pleuro-pneumonia, the unit of com-

plement (M.H.D.) is ascertained by titrating the complement with red blood cells, sensitised by more than a single unit of amboceptor, it is possible to arrive at a unit of complement, which is just large enough to effect complete haemolysis of the sensitised red blood cells, in the case of a negative serum, but which at the same time is small enough to be more or less completely fixed by the antigen—antibody combination in the case of a positive serum.

This "overloading" of the haemolytic system with amboceptor cannot be increased beyond certain limits. While a slight increase of the amboceptor factor is a decided advantage, the increase must not exceed two complete units, for it has been found that, owing to the feeble nature of the fixation which occurs in contagious pleuro-pneumonia, a large increase in the amount of amboceptor in the haemolytic system tends to dissociate some of the complement already held by the antigen-positive serum combination. In consequence of this, a large excess of amboceptor only tends to shorten the time required for haemolysis to occur.

It has been found that the best results have been obtained by using 1½ units of amboceptor to sensitise the red blood cells, and then to titre the complement against one unit of these over sensitised cells, in order to determine the absolute M.H.D. of complement required for haemolysis.

Technique of the Complement Fixation Test for Contagious Pleuro-Pneumonia.

The method of carrying out the test is as follows:—

0.5 c.c. of a 5 per cent. suspension of sheep's red blood cells equals 1 unit. Against this unit, the haemolytic amboceptor is titrated, and it is found that 0.5 c.c. of 1 in 1000 dilution equals one unit of haemolytic amboceptor. Each unit of red blood cells is sensitised by 1½ units of haemolytic amboceptor, by mixing together the necessary units of amboceptor and red blood cell suspension, and allowing them to stand for one hour. The mixture is then centrifuged, and the clear fluid pipetted off. The sensitised red blood cells are then washed with saline solution, and are resuspended in saline in order to make a 5 per cent. suspension. 0.5 c.c. sensitised red blood cells (5 per cent. suspension) equals one unit.

The complement (guinea-pig's serum, 1 in 10), is now titrated against one unit of sensitised red blood cells, by testing it in

decreasing doses from 0.5 c.c. downwards. The smallest amount of complement necessary to produce complete haemolysis in 1/4 hour is noted. This amount varies in different complement samples, but is usually approximately 0.15 c.c. of a 1 in 10 dilution. The estimation of the minimum haemolytic dose (M.H.D.) of complement must be decided with absolute accuracy, any quantity in excess of the absolute M.H.D. being sufficient to render the reading of the final test exceeding difficult, if not impossible. The necessity for only adding the absolute M.H.D. of complement to the final test, will be obvious when the action of conglutinin is considered.

When the M.H.D. of complement has been determined, the haemolytic system is standardised, and against this standardised haemolytic system the other ingredients of the test are titrated in order to determine the proper quantity of each to employ.

The antigen is titrated to ascertain its anticomplementary unit. The smallest amount which inhibits haemolysis is the anti-complementary unit. Half that amount, or even less, is used in the final test.

After the titre of the antigen has been determined in the above-mentioned manner, and when carrying out subsequent tests in which fresh complement of an unknown titre has to be used, it is convenient, when ascertaining the M.H.D. of complement, to titre it in the presence of the unit of antigen previously determined.

Each bovine test serum is similarly titrated in order to determine the maximum amount which does not inhibit haemolysis. It was thought at first that a definite amount of test serum could be taken as a standard unit, and, provided the quantity of the standard unit was small enough, it would render a titration unnecessary. It was found, however, that the test sera differed very much in their behaviour towards complement, and that for accuracy in the final result it was necessary to titrate each serum separately. The controls in this titration are most important, and the unit of bovine serum to be determined is the largest amount which allows complete haemolysis to occur in the tube containing it in the same minimum time as complete haemolysis takes place in a tube containing only saline solution plus the titrated units of complement and sensitised red blood cell suspension.

The amount of each test serum to be used in the final test must be so determined that the amount employed is slightly less

than the largest amount which has been shown not to interfere with the complete haemolysis of the standardised haemolytic system it has been titrated against.

Having ascertained the exact quantity of each ingredient to employ, the final test is now set up as follows, assuming for example that the quantity of each ingredient shown below is the titrated unit.

	Tube.	Antigen, 1 in 10.	Inactivated ox serum, 1 in 10.	Complement 1-10.	Saline.	Sensitised R.B.C. 5% suspension.
GROUP I.—						
Serum No. I (known positive serum)	1	·15	·1	·15	1·6	·5
	2	—	·1	·15	1·75	·5
	3	—	·1	—	1·9	·5
GROUP II.—						
Serum No. II. (known negative serum)	4	·15	·1	·15	1·6	·5
	5	—	·1	·15	1·75	·5
	6	—	·1	—	1·9	·5
GROUP III.—						
Serum No. III. (suspected serum to be tested)	7	·15	·1	·15	1·6	·5
	8	—	·1	·15	1·75	·5
	9	—	·1	—	1·9	·5
Antigen control	10	·15	—	·15	1·7	·5
Complement control	11	—	—	·15	1·85	·5
Saline control	12	—	—	—	2·	·5

Incubate for one hour at 37°C. — Incubate at 37°C.

The first tube in each group of three contains antigen, and it is therefore the tube in which the particular serum is being tested. All other tubes in the series are controls.

The second tube in each group of three is the serum control for the particular serum of the group. This tube must show complete haemolysis before the reaction of the particular serum in the test can be recorded.

The third tube in each group of three is also a serum control, but in this tube complement is omitted. This tube serves as a control to show that the particular ox serum is not in itself haemolytic for the unit of sensitised R.B.C. No haemolysis should occur in this tube. After the first incubation for one hour has taken place, one unit of sensitised red blood cells is added to each tube, and the rack is then replaced in the incubator. The duration of the final incubation period varies usually from 10 to 30 minutes, and the tubes have to be carefully watched in order to note the appearance of haemolysis. Complete haemolysis must have taken place in both the antigen and compelment

control tubes (tubes 10 and 11), before a reading of the tubes containing the test serum is made.

If both the antigen and complement controls show complete haemolysis, the serum control tube of each particular serum being tested (tubes 2, 5, and 8) is next examined. If this tube shows complete haemolysis, its companion tube containing antigen is next examined. If the reaction is positive, this antigen tube should show no haemolysis at the time when its serum control tube shows complete haemolysis. If the reaction is negative, complete haemolysis will be shown in the antigen tube as well as in the serum control tube at the same time.

Border-line reactions sometimes occur, but their number is usually small, and certainly not greater than the number of such reactions obtained with complement fixation tests in other diseases.

It is important in reading the final result to observe the exact time required for haemolysis to occur in the controls, for, if the reading of the test is delayed it is possible for a serum to have shown fixation, and the fixation to become masked by a subsequent haemolysis. This subsequent haemolysis is due to the conglutinin present in the bovine serum, reinforcing the small amount of unfixed complement, and assisting that amount of complement to haemolyse more or less completely the sensitised red blood cells. This ability of conglutinin to augment the haemolytic power of a dose of complement, too small in itself to produce haemolysis, is strikingly shown in the experiment set out in detail on pages 44-45.

Results of Complement Fixation Tests.

Up to the time of writing this report, 63 different bovine sera have been tested for complement fixation in the manner just described. Of this number, 14 have given definite positive reactions, and 47 have given definite negative reactions, while two border line and doubtful reactions have been recorded.

In each case the reaction given to the test has been checked by a post-mortem examination of the animal supplying the particular serum tested.

A number of the animals tested were dairy cows from farms which were quarantined owing to the presence of contagious pleuro-pneumonia on them. These animals had each received a prophylactic injection of virus subcutaneously in the tail some

time prior to being tested. The length of time between the prophylactic injection, and the test varied from 15 days with one lot of animals to 6 months with another lot. It is worthy of note that in all cases where an animal which had been injected with virus subcutaneously in the tail was tested, the reaction to the test was negative, excepting in those cases where lesions of contagious pleuro-pneumonia could be demonstrated in the lungs on post-mortem examination, in which cases the reactions to the tests were positive.

Two of the animals tested had each reacted so violently to the prophylactic injection of virus in the tail, that portion of the tail was necrotic at the time that the blood sample was taken for testing. These two animals, on testing, gave negative complement fixation reactions, and showed no lesions of contagious pleuro-pneumonia in the lungs on post-mortem examination.

The sera tested, the results of the tests, and the post-mortem findings in each case can be tabulated as follow:—

Serum Number.	Result of Complement fixation test.	Result of P.M. examination of the animal supplying the test serum.
40	Positive	Active C.P.P., involving practically the whole of one lung
41	Negative	Hydatids in lung—no lesions of C.P.P.
42	Negative	No lesions of C.P.P.
43	Negative	No lesions of C.P.P.
44	Negative	Tuberculosis in lungs and lymph glands. No lesions of C.P.P.
45	Negative	No lesions of C.P.P.
46	Negative	No lesions of C.P.P.
47	Negative	No lesions of C.P.P.
48	Positive	Small chronic encapsulated lesion of C.P.P. in one lung. Tuberculosis in pharyngeal lymph glands.
49	Positive	Active C.P.P. involving whole of right lung.
50	Positive	Small and very early lesion of C.P.P. on margin of left lung.
51	Positive	Active C.P.P. involving practically the whole of the left lung.
52	Doubtful	Calf 2 days old. No lesions of C.P.P. Mother was infected (see No. 49).
53	Positive	No lesions of C.P.P. in the lungs. Animal had not been inoculated.
54	Negative	No lesions of C.P.P.
55	Negative	Extensive hydatid infestation. No lesions of C.P.P.

Serum Number.	Result of Complement fixation test.	Result of P.M. examinations of the animal supplying the test serum.
56	Negative	No lesions of C.P.P.
57	Negative	No lesions of C.P.P.
58	Negative	No lesions of C.P.P.
59	Negative	No lesions of C.P.P.
60	Positive	Old encapsulated lesion in left lung.
61	Negative	No lesions of C.P.P.
62	Positive	Small active lesions right lung.
63	Negative	No lesions of C.P.P.
64	Negative	No lesions of C.P.P.
65	Negative	No lesions of C.P.P.
66	Positive	Small and early lesions of C.P.P. on margin right lung.
67	Negative	No lesions of C.P.P. Tuberculosis supra mammary gland and in udder.
68	Positive	Old chronic lesion in left lung with necrosis.
69	Negative	No lesions of C.P.P. Actinomycotic infection of the udder.
70	Negative	No lesions of C.P.P. Old adhesions between left lung and diaphragm.
71	Positive	Small active lesion in left lung, extensive exudate in chest cavity.
72	Negative	No lesions of C.P.P.
73	Negative	No lesions of C.P.P. Perforation by piece of wire through rumen and diaphragm to lung with adhesions and pus.
74	Negative	No lesions of C.P.P.
75	Negative	No lesions of C.P.P.
76	Negative	No lesions of C.P.P.
77	Negative	No lesions of C.P.P.
78	Negative	No lesions of C.P.P.
79	Negative	No lesions of C.P.P., extensive hydatid infection in lungs and liver.
80	Negative	No lesions of C.P.P.
81	Negative	No lesions of C.P.P.
82	Positive	Active lesions involving practically whole of one lung, with adhesions between costal and pulmonary pleurae. Extensive exudate in chest cavity.
83	Negative	Tuberculosis in lungs and lymph glands. No C.P.P.
84	Negative	No lesions of C.P.P.
85	Negative	Old adhesions between costal and pulmonary pleurae. No C.P.P.
86	Doubtful	No lesions of CP.P. in lungs.
87	Positive	Large chronic encapsulated lesion in one lung, with necrosis. Adhesions between lung and diaphragm.

Serum Number.	Result of Complement fixation test.	Result of P.M. examinations of the animal supplying the test serum.
88 -	-	
89 -	-	
90 -	Negative -	No lesions of C.P.P.
91 -	-	
92 -	-	
93 -	Negative -	Tuberculosis in lungs and lymph glands. No C.P.P.
94 -	Positive -	Active lesions of C.P.P. in left lung, and fluid in chest cavity.
95 -	-	
96 -	-	
97 -	-	
98 -	Negative -	No lesions of C.P.P.
99 -	-	
100 -	-	
101 -	-	
102 -	-	

The positive reaction given by No. 53 is difficult to explain. The lungs of this animal were submitted to a searching post-mortem examination, but no lesions of contagious pleuro-pneumonia could be demonstrated in them.

No. 52 was a calf two days old. The serum of this animal gave a border line reaction, which it was impossible to interpret, either as negative or positive. This reaction might be explained by the fact that this animal, although not affected with contagious pleuro-pneumonia, was the progeny of an infected mother, who showed active lesions of the disease on post-mortem examination. No. 86 was an apparently healthy bull, and no lesions of contagious pleuro-pneumonia could be demonstrated in the lungs on post-mortem. This bull gave a border line reaction to the test, which could not be interpreted, either as negative or positive.

It will thus be seen that, with the exception of the three reactions specially quoted, the complement fixation test has proved reliable in differentiating between animals which are, and which are not, affected, with contagious pleuro-pneumonia.

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During the progress of these latter complement fixation tests with alcoholic extracts as antigen, my attention was directed to an abstract, published in the *Tropical Veterinary Bulletin*, Vol. 8 (March 30th, 1920), p. 55, of an article, "Feststellung Der

Lungenseuche Mit Hilfe Der Kemplementablenkung," by Titze and Gieze, published in the *Berliner Tierarztliche Wochenschrift*, No. 32, Vol. 35 (August, 1919, pp. 281-282). Unfortunately, I have not been able to see the original article by Titze and Gieze, but it would appear from the abstract already referred to that, while there are many points in common in regard to the reaction described by Titze and Gieze, and that which I have just described, they differ in certain very material points. Titze and Gieze used as antigen tissue the fresh lung and bronchial lymph glands taken from an infected animal. With this tissue they prepared:—

(1) A watery extract by boiling, afterwards centrifuging to obtain a clear solution, which was then made isotonic by the addition of the necessary quantity of sodium chloride.

(2) An alcoholic extract which was afterwards cleared in the centrifuge, and diluted with from 10 to 50 parts of saline solution. Since reading the abstract of Tietze and Gieze's work I have prepared, and tried alcoholic extracts of diseased lung. While it is possible to obtain complement fixation with such extracts, the results obtained with them have not been satisfactory, and in certain instances have been quite unreliable. I am thoroughly of the opinion that sub-epidermal tumour tissue is better material from which to prepare the antigen. Alcoholic extract of dried sub-epidermal tumour tissue gives uniform fixation, and thoroughly reliable results, if the technique laid down is carefully followed.

It is probable that an alcoholic extract prepared from fresh subepidermal tumour tissue will give equal, if not better, results than that prepared from dried tissue, but, at the present time, I have not had an opportunity of testing it.

Titze and Gieze mention the haemolysis which often occurs after a positive result, and quite correctly refer to the necessity of carefully titrating the complement, in order to ascertain the absolute M.H.D. In the method of complement titration we differ in technique, but either method gives good results. While mention is made of the fact that haemolysis usually follows a positive result, Titze and Gieze apparently do not attempt to explain the phenomena which bring about that haemolysis.

The tests which I have conducted, in order to determine the influence of conglutinin on haemolysis in the complement fixation test in contagious pleuro-pneumonia, offer an obvious explanation for its occurrence. It seems quite evident that the

conglutinin present in the test serum (bovine serum) reinforces a small amount of complement, which has not been fixed, and permits haemolysis of the sensitised red blood cells to occur.

The variation in time required for this haemolysis to occur in the test is due to the amount of complement and conglutinin present and capable of acting in combination to bring about haemolysis.

The amount of conglutinin present in bovine sera differs very considerably. It is on account of this variability in the conglutinin content of the test sera that I prefer to titrate each test serum separately, and thus arrive at the proper quantity of each to use in the final test.

Titze and Gieze apparently do not do this, but adopt as a standard amount approximately 0.1 c.c. of a 1 in 10 dilution. This latter method I have proved to be particularly prone to produce reactions which are not as sharply defined as when the test serum has been titrated, and the proper quantity, revealed by the titration, employed in the final test. With certain sera, such as those which give border line reactions, the proper titration of the test serum is an absolute essential to a reliable result in the final test, for an amount of serum either short of, or in excess of the proper quantity, would be sufficient to produce a totally different result to the proper one.

Experiments to Remove Conglutinin from Bovine Serum.

The results of the complement fixation experiments outlined in the previous chapter have proved that complement fixing antibodies are present in the sera of animals affected with contagious pleuro-pneumonia. It has also been demonstrated that by adopting a special technique, it is possible to test a serum for the presence or absence of these antibodies.

The presence of conglutinin in bovine serum has made the elaboration of a special technique necessary for this test, which requires very careful manipulation in all stages, and very careful observation in the final stage in order to guard against error in the final reading.

If conglutinin could be eliminated altogether from the bovine serum, it was thought that the test with sera deprived of conglutinin could be much simplified, and the necessity for careful and exact observation of the time required for haemolysis to occur in the final stage could be dispensed with.

Experiments were accordingly made in order to remove all traces of conglutinin from various bovine sera, which were afterwards tested to determine the effect the experiment had produced on their complement fixing properties.

Bordet and Gay (3) have shown that conglutinin is not destroyed by heating bovine serum at a temperature of 56°C. To attempt to destroy conglutinin by heating at higher temperatures would probably render the serum useless for test purposes afterwards, consequently some other method had to be adopted in order to get rid of it. If inactivated bovine serum plus complement is added to a saline suspension of red blood cells previously sensitised by their specific sensitiser, conglutination of the red blood cells takes place more or less immediately on mixing, and in the reaction produced the conglutinin becomes used up. This conglutination is followed after a varying time by haemolysis of the red blood cells, the time depending on the source and activity of the complement used in the experiment. If, then, when conglutination occurs in such an experiment, and before haemolysis has time to occur, the mixture is rapidly centrifuged, the supernatent fluid can be pipetted off from the deposit of red blood cells, and will be found to contain little or no conglutinin; the absence of conglutinin depending on the degree of saturation of the bovine serum by the red blood cells. Thus it is possible to deprive a bovine serum of its conglutinin.

Certain bovine sera, which had been taken from animals known to be affected with active lesions of contagious pleuro-pneumonia, were used for experimental purposes. The experiments carrid out were as follows:—

Experiment 1.—To 10 c.c.s. of a 5 per cent. suspension in saline of washed red blood cells of a sheep, which have been previously sensitised by their specific amboceptor, 1 c.c. of fresh horse serum is added. Mix, and allow to stand at room temperature for one hour. Haemolysis does not occur in one hour, because the horse serum—although it contains complement—contains only a very weak complement. Centrifuge the mixture, and carefully remove the fluid from the deposit of cells. The cells are then washed in saline solution, which is afterwards removed. Add then to the deposit of cells, 1 c.c. of saline solution, and 1 c.c. inactivated bovine serum (No. 51 positive). Conglutination of the red blood cells takes place almost immediately on mixing. Carefully remove the supernatent fluid, and dilute it 1 part in 4 of saline solution, thus forming a 1 in 10 dilution of the original amount

of bovine serum used. The complement fixing properties of this diluted serum are then tested. As a control, a 1 in 10 dilution of serum (No. 51) from the same bulk sample, but which has not been treated for extraction of conglutinin, is also tested.

The untreated serum gives a strongly positive reaction, when tested, whereas the treated serum gives a negative reaction. The treated serum is found to be devoid of conglutinin, but in the process of extracting the conglutinin, the specific complement fixing antibodies have also disappeared. It is thus apparent that serum so treated is rendered useless for the purpose of a complement fixation test for contagious pleuro-pneumonia.

Experiment 2.—To 3.5 c.c.s. of a 10 per cent. suspension of the washed red blood cells of a guinea-pig there is added 1 c.c. saline solution, and 0.5 c.c. of fresh horse serum. (Horse serum contains a natural sensitiser for guinea-pig's cells, the fresh horse serum also contains complement). Mix thoroughly by shaking, and allow to stand at room temperature for one hour, then centrifuge. Pipette off the fluid, and add 2 c.c.s. saline solution to the deposit of cells. Mix and centrifuge. Pipette off the saline solution, and add to the deposit of cells, 2 c.c.s. saline solution, and 0.5 c.c. inactivated bovine serum (Serum No. 94, positive). Mix, and allow to stand at room temperature for 30 minutes, then centrifuge. Carefully remove the fluid from the deposit of cells, and add to this fluid 2.5 c.c.s. of saline solution, thus forming a 1 in 10 dilution of the original amount of bovine serum used. This dilution of treated bovine serum is then tested for its complement fixing properties. It gives a feebly positive reaction, whereas untreated serum No. 94, from the same bulk sample, gives a strongly positive reaction. It is found, on testing, that the experiment to remove the conglutinin from the serum so treated, has failed to remove it completely, although the greater bulk of it has been extracted.

Experiment 3.—To 10 c.c.s. of a 10 per cent. suspension in saline solution of the washed red blood cells of a goat there is added 1 c.c. of fresh unheated bovine serum (Serum No. 94, positive). (Bovine serum contains a natural sensitiser for goat's cells, and fresh bovine serum also contains complement). Allow the mixture of serum and cells to stand at room temperature for 20 minutes. Conglutination of the red blood cells takes place a few minutes after the mixture is made. Centrifuge and carefully pipette off the supernatent fluid from the deposit of red blood cells. The supernatent fluid is bovine serum diluted 1 in 10

with saline, and it should now contain no conglutinin. The absence of conglutinin can be tested for by adding this supernatent fluid to another amount of washed goat cells, and noting whether any conglutination occurs. It has been found that one application of 10 c.c.s. of a 10 per cent. suspension of goat's cells was sufficient to extract all the conglutinin from 1 c.c. of any one of the bovine sera, which have been treated in this way. The bovine serum dilution, after the conglutinin has been extracted, is then heated in a water bath at 56°C. for 30 minutes, to destroy any complement which may remain. On submitting this treated bovine serum to a test for complement fixation, it is found that it gives a negative reaction, whereas untreated serum No. 94 gives a strongly positive reaction.

From the results obtained in the experiments outlined above it is obvious that, while it is possible to extract conglutinin from bovine serum, the extraction of conglutinin also brings about the disappearance of the specific antibodies; consequently, sera so treated are useless for the purposes of complement fixation tests for the diagnosis of contagious pleuro-pneumonia.

Conclusion.

(1) Agglutinins could not be demonstrated, in the serum taken from bovines known to be affected with contagious pleuro-pneumonia, by the usual macroscopic and microscopic methods of testing for agglutinins. Therefore an agglutination test apparently has no value as a means of differentiating between animals which are, and which are not, affected with the disease.

(2) Complement fixing antibodies are present in the serum of animals affected with the disease, and a complement fixation test can be used to differentiate infected from non-infected animals.

(3) In order to obtain reliable results with the complement fixation test, the special technique outlined in the proceeding pages must be closely followed in every particular.

(4) The main difficulty in carrying out complement fixation for the diagnosis of contagious pleuro-pneumonia is to prevent errors arising in the test owing to the presence of conglutinin in the test serum.

(5) Bovine serum, from which the conglutinin has been extracted by adopting the methods outlined in the preceding pages, is rendered useless for the purposes of a complement fixation test owing to the inability to prevent, with extraction of the con-

glutinin, the extraction also of the specific complement fixing antibodies.

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